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Discovery of serum proteomic biomarkers for prediction of response to infliximab (a monoclonal anti-TNF antibody) treatment in rheumatoid arthritis: an exploratory analysis

Ortea, Ignacio ; Roschitzki, Bernd ; Ovalles, Juan Gabriel ; Longo, Javier López ; de la Torre, Inmaculada ; González, Isidoro ; Gómez-Reino, Juan J ; González, Antonio

Abstract: Biologics such as TNF antagonists are a new class of drugs that have greatly improved Rheumatoid Arthritis (RA) treatment. However, for unknown reasons, individual patients with RA respond to one of these drugs but not to others even those targeting the same molecule. Methods to predict response are sorely needed because these drugs are currently selected by trial and error, what is very inefficient and prejudicial for the patient and the healthcare system. Here, we have explored the discovery of protein biomarkers in serum from patients treated with infliximab, one of the major anti-TNF drugs. The study was based in a quantitative proteomics approach using 8-plex iTRAQ labeling. It combined depletion of the most abundant serum proteins, two-dimensional LC fractionation, protein identification and relative quantification with a hybrid Orbitrap mass spectrometer. This approach allowed the identification of 315 proteins of which 237 were confidently quantified with two or more peptides. The detection range covered up to 6 orders of magnitude including multiple proteins at the ng/mL level. A new set of putative biomarkers was identified comprising 14 proteins significantly more abundant in the non-responder patients. The differential proteins were enriched in apolipoproteins, components of the complement system and acute phase reactants. These results show the feasibility of this approach and provide a set of candidates for validation as biomarkers for the classification of RA patients before the beginning of treatment, so that anticipated non-responders could be treated with an alternative drug.

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**Discovery of serum proteomic biomarkers for prediction of
response to infliximab (a monoclonal anti-TNF antibody)
treatment in Rheumatoid Arthritis: an exploratory analysis**

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Abstract

Biologics such as TNF antagonists are a new class of drugs that have greatly improved Rheumatoid Arthritis (RA) treatment. However, for unknown reasons, individual patients with RA respond to one of these drugs but not to others even those targeting the same molecule. Methods to predict response are sorely needed because these drugs are currently selected by trial and error, what is very inefficient and prejudicial for the patient and the healthcare system. Here, we have explored the discovery of protein biomarkers in serum from patients treated with infliximab, one of the major anti-TNF drugs. The study was based in a quantitative proteomics approach using 8-plex iTRAQ labeling. It combined depletion of the most abundant serum proteins, two-dimensional LC fractionation, protein identification and relative quantification with a hybrid Orbitrap mass spectrometer. This approach allowed the identification of 315 proteins of which 237 were confidently quantified with two or more peptides. The detection range covered up to 6 orders of magnitude including multiple proteins at the ng/mL level. A new set of putative biomarkers was identified comprising 14 proteins significantly more abundant in the non-responder patients. The differential proteins were enriched in apolipoproteins, components of the complement system and acute phase reactants. These results show the feasibility of this approach and provide a set of candidates for validation as biomarkers for the classification of RA patients before the beginning of treatment, so that anticipated non-responders could be treated with an alternative drug.

Keywords: rheumatoid arthritis, infliximab, serum biomarkers, response prediction.

Running title: Biomarkers for prediction of response to infliximab in RA.

1 **Introduction**

2
3 Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of complex
4 etiology comprising genetic and environmental factors that is characterized by
5 inflammation in multiple joints [1]. Left without treatment, it progresses to disability,
6 deformities due to bone erosion and life shortening. RA prevalence is about 1% of the
7 world population. Classical treatments are still commonly used, but they are not
8 sufficiently effective for many patients. In the last decade, new drugs became available
9 in the group of biologics (monoclonal antibodies, soluble receptors or other complex
10 molecules targeting specific players in the disease process). The first that were available
11 for RA treatment were the tumor necrosis factor (TNF) antagonists. Among them,
12 infliximab, a chimeric antibody comprising a human IgG1 constant fraction and a
13 murine variable region targeting membrane and soluble TNF [2], has become one of the
14 biologics most commonly used in RA.

15 Biologics have greatly improved RA treatment but none of them is effective in
16 all patients. For unknown reasons, about a third of the patients in whom one of these
17 drugs is assayed fail to show significant improvement. These patients can respond to an
18 alternative biologic targeting the same or a different molecule [3]. Currently, clinical or
19 laboratory methods for the prediction of patients response are not available. Therefore,
20 the only approach to select biologics for a particular RA patient is by trial and error.
21 This approach is associated with notable inefficiency and prejudices because
22 responsiveness can only be assessed after three to six months of treatment. During this
23 time, patients suffer uncontrolled disease with the potential of irreversible damage, and
24 the healthcare system expends large amounts in ineffective drugs. Thus, it is necessary
25 to find biomarkers that make possible the identification of non-responder patients in
26 advance, to treat them with an alternative drug from the beginning. Many studies have
27 already tried to identify this type of biomarkers in the genetics, functional genomics,
28 proteomics, autoantibody and clinical fields, but no reproducible and informative
29 findings have yet been reported [4].

30 Very few proteomic studies have attempted to identify biomarkers for prediction
31 of response to biologics in RA. A couple of studies analyzed selected cytokines or
32 cytokines plus RA autoantibodies showing that some of them were associated with
33 clinical response to the TNF antagonist etanercept [5,6]. By contrast, not a single
34 cytokine was associated with response to a different biologic, rituximab, in a similar

study [7]. The unique previous agnostic proteomic study was done by Trocmé et al. [8]. These authors used SELDI-TOF technology to identify plasma biomarkers for prediction of response to infliximab. Six potential biomarkers were detected, although only two proteins were identified. None of the previous studies have been independently replicated. This paucity of proteomic research on biomarkers for prediction of response contrasts with the multiple studies searching RA diagnostic and prognostic biomarkers [9-20], and with several proteomic studies monitoring the changes that take place after the administration of different drugs [7,16,21,22].

Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) is a quantitative proteomic approach ideally suited for biomarker discovery. It provides quantification, identification and multiplexing in a single assay. However, it has been scarcely used to study human serum and plasma. This is unfortunate because serum and plasma are informative for many diseases, especially for systemic diseases like RA, and easily available. The latter is very important when validating the potential biomarkers and also for their future widespread use. For example, patients with RA are not routinely subjected to synovial tissue biopsies and it will pose significant difficulties to implement them for drug selection. Unfortunately, discovery of biomarkers among the serum or plasma proteins is limited by their great complexity and wide dynamic range. Protein concentrations extend for more than 11 orders of magnitude with the top 10 most abundant plasma proteins accounting for ~ 90% of the total proteins [23]. Disease biomarkers are usually present at low concentrations (~ ng/mL) [23], being masked by higher abundance proteins in 2-DE and being blurred in MS due to competitive ionization and signal suppression. Therefore, the quantitative and qualitative analysis of low abundance proteins is challenging. To overcome these problems, there is a need for (i) prefractionation methods to specifically remove the high abundance proteins; (ii) good separation techniques to further decrease protein complexity; and (iii) MS equipments with high sequencing speed and sensitivity. In this study, we have explored the performance of an approach including these characteristics. It was applied to the discovery of biomarkers for prediction of response in serum of patients with RA that had been prospectively evaluated during treatment with infliximab. The proteomics approach comprised an immunodepletion prefractionation step, a thorough 2-D LC fractionation and quantification of the differentially isotopic labeled peptides by MS/MS. Results were satisfactory because this approach allowed the identification of a large number of proteins, covering a wide dynamic range and including many proteins

1 in the ng/mL level, and identifying 14 putative biomarkers for prediction of response to
2 infliximab that are consistent with our knowledge of the disease.

3 4 **Materials and Methods**

5 6 *Ethics approval*

7
8 The project was approved by the Ethics Committee for Clinical Research of
9 Galicia and carried out according to the Helsinki Declaration Principles. All
10 participating subjects gave their written informed consent.

11 12 *Sample collection*

13
14 Patients with RA according to the American College of Rheumatology (ACR)
15 classification criteria [24] from a single center (Gregorio Marañón Hospital, Madrid,
16 Spain) were enrolled in the study. All were naive for any biologics before the start of
17 the enrollment period. Blood was collected into 8 ml Vacuette Z Serum Sep Clot
18 Activator tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) before starting
19 infliximab administration, left to clot at room temperature for 2 hours and then
20 centrifuged at 3000 g for 10 min. The collected serum was aliquoted and stored at -80°C.
21 Infliximab (Remicade; Centocor Inc., Malvern, PA) was given following the standard
22 dose and administration schedule. Clinical response was determined 6 months after
23 infliximab initiation according with the European League Against Rheumatism
24 (EULAR) criteria based in the Disease Activity Score 28 (DAS28) [25]. Only patients
25 classified as non-responder (NR, n = 4) or good responder (R, n = 4) were compared to
26 increase the chances of finding differences. The intermediate class of moderate
27 responders was excluded.

28 29 *Immunoaffinity depletion of high-abundance proteins*

30
31 The six most abundant proteins in serum were depleted using the Hu-6 Multiple
32 Affinity Removal System kit (Agilent Technologies, Wilmington, USA) following
33 manufacturer's instructions. Afterwards, the remaining proteins were concentrated using
34 5000 MWCO spin concentrators (Agilent Technologies) and acetone precipitation. The

1 resulting air-dried pellets were dissolved in 0.5 M triethylammonium bicarbonate
2 (TEAB) pH 8.5 buffer and protein concentration was measured.

3 4 *Protein digestion and iTRAQ labeling*

5
6 For each sample, 40 µg of protein, at 1 mg/mL in 0.5 M TEAB pH 8.5 and 0.1%
7 SDS, were reduced with 5 mM tris-(2-carboxyethyl) phosphine (TCEP) at 60°C for 1
8 hour and cysteine-blocked with 10 mM methyl methanethiosulfonate (MMTS) at RT for
9 10 min. The proteins were then digested with Sequencing Grade Modified trypsin
10 (Promega, Madison, WI, USA) at a trypsin-to-protein ratio of 1:50, at 37°C for 6 hours.
11 Each digest was labeled at 25°C for 3 h with one of the 8-plex iTRAQ reagents
12 previously solubilized in 100 µl isopropanol, according to the manufacturer's
13 instruction (AB Sciex, Framingham, MA, USA). The labeling reactions were stopped
14 by adding phosphoric acid to reach pH below 4.0, and all iTRAQ-labeled samples were
15 combined into one tube. Tryptic peptides were then dried by centrifugal evaporation.

16 17 *Peptide fractionation with SCX chromatography*

18
19 Peptides were fractionated using a PolyLC SCX Polysulphoethyl A 200 mm x
20 2.1 mm, 5 µm, 200 Å column (PolyLC, Columbia, MD, USA), on a high-pressure LC
21 pump (1100-series, Agilent Technologies). Dried peptides were reconstituted in 600 µl
22 buffer A, the pH was adjusted to 2.7 with H₃PO₄. The flow rate was kept at 0.2 ml/min,
23 and the sample was fractionated using a two-buffer system (buffer A, 7mM KH₂PO₄ in
24 25% ACN pH 2.7; buffer B 7mM KH₂PO₄, 500 mM KCl in 25% ACN pH 2.7). The
25 gradient employed was 0% B for 20 min, 0% to 5% B in 5 min, 5% to 35% B in 35 min,
26 35% to 100% B in 10 min, 100% B for 5 min, and then 0% B for 25 min. Eluted
27 peptides were monitored at 214 and 280 nm and collected from 20-25.7 min, 25.7-31.3
28 min, 31.3-37.0 min, then every 2.4 min until 95 min. A total of 27 fractions were
29 collected and then dried. An aliquot of 0.5 µl of each fraction was desalted by C18
30 ZipTip tips (Millipore, Billerica, MA, USA) and analyzed by MALDI-TOF-TOF (4800,
31 AB Sciex) MS to check its peptide complexity. The first three fractions were discarded,
32 and the other 24 fractions were mixed in 12 final fractions according to their complexity.

33 34 *Nano-reverse-phase LC-MS/MS*

Combined fractions were desalted onto C18 spin tips (StageTips, Thermo Fisher Scientific, Waltham, MA, USA) lyophilized and dissolved on buffer A. Analysis by nanoHPLC-MS/MS was done using a NanoLC-Ultra system (Eksigent, Dublin, CA, USA) coupled to an Orbitrap Velos hybrid mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). The separation was performed on a inhouse-made tip column (75 μ m id x 8 cm) packed with Magic RP C18 AQ, 200A, 3 μ m beads (Bischoff GmbH, Leonberg, Germany), at a flow rate of 250 nl/min. Water with 1% ACN and 100% ACN, both containing 0.2% formic acid, were used as solvents A and B, respectively. Peptides were loaded on the column for 16 min with 2% of solvent B and a flow rate of 500 nl/min. Peptide elution was started using the following gradient of solvent B: 0 to 1 min 2% to 10% B, 1 to 65 min 10% to 30% B, 65 to 70 min 30% to 45% B, 70 to 74 min 45% to 97% B. The column was washed for 7 min with 97% B and then re-equilibrated for 9 min with 2% solvent B before the next run. The mass spectrometer was operated in data-dependent mode with the following ion scanning parameters: survey MS scan in FT mode from 300 to 2000 m/z (resolution 30000), followed by top eight peaks collision induced dissociation (CID) fragmentation (isolation width 2 m/z, normalized collision energy 35%) for identification, plus the same top eight peaks higher-energy collisional dissociation (HCD) fragmentation (isolation width 2 m/z, normalized collision energy 52%) and readout in the FT analyzer (resolution 7500) for quantification. Fragmented peptide masses were set in dynamic exclusion for 60 s and singly charged ions were excluded from MS/MS analysis. To improve sensitivity of the MS/MS analysis for peptides of low-abundance proteins, each fraction was run a second time excluding previously fragmented precursors.

Protein identification and protein relative abundance

Peptide and protein identification was performed with ProteinPilot software v4.0 (AB Sciex) and the Paragon algorithm [26] by comparison with the human Swissprot/TrEMBL database (downloaded in December 2011). Paragon method parameters were: peptide labeled with iTRAQ 8plex, fixed modification of methyl methanethiosulfonate on Cys (+46 Da), digestion with trypsin, instrument Orbi/FT MS (1-3ppm) LTQ MS/MS, and ID focus on biological modification. Proteins having at least one peptide above the 95% confidence level as determined by Protein Pilot were

recorded. False discovery rates (FDR) were estimated using a concatenated target-decoy database [27]. For the estimation of the protein abundance ratio, the intensities of iTRAQ reporter ions for each MS/MS spectra were extracted from ProteinPilot and the sum ratio for each protein was calculated across the spectra matched to the corresponding peptides. Data were normalized for loading error by bias corrections using ProteinPilot. The statistical significance of the differences between the means for each group (R vs. NR) was determined on the transformed data (arc sin hyperbolic) using the two-tailed t-test. Threshold for significance was set at $p < 0.05$. A receiver operation characteristic (ROC) curve analysis was performed for each protein to obtain the area under curve (AUC). AUC was used as the summary statistic reflecting the overall predictive accuracy of each protein [28]. A model or test with perfect discriminatory ability will have an AUC of 1.0, while a model of random outcomes will have an AUC of 0.5. Interactions and pathways of proteins with fold differences between NR and R patients higher than 1.5 were analyzed with Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA).

Results and Discussion

Figure 1 shows the discovery-driven 8-plex iTRAQ workflow used in this study. This workflow combines depletion of the highest abundance serum proteins, intensive fractionation of the depleted serum, and MS/MS based identification and quantification of the fractionated proteins. In the first step, sera were immunodepleted from the six highest abundance proteins on a human MARS-6 spin column. Reproducibility and protein recovery of this step were evaluated by 1-DE (Figure 2) and by total protein quantification. These analyses revealed uniform reduction of major protein bands and compensatory increases in other bands of lower concentration together with significant reduction of the total protein concentration. The mean protein recovery rate was 11.5%, which is in agreement with the column's manufacturer specifications. Subsequent MS analysis showed that this depletion step was effective because our protocol allowed the identification with strict criteria (at least 2 peptides and 95% confidence) of medium and low concentration proteins. Examples of identified proteins that are in the ng/mL range according with the bibliography were hepatocyte growth factor activator, sex hormone-binding globulin, alpha synuclein and retinoic acid receptor responder protein [29-32]. However, our protocol was not able to identify very rare proteins in the pg/mL

range. Since concentrations of the most abundant identified proteins are in the mg/mL level, the workflow used in this study allowed the detection of serum proteins with a dynamic range of up to 10^6 . A wide detection range is a necessary characteristic for effective proteomic analysis of serum given the complexity and heterogeneity of this biologic fluid.

Identification of serum proteins

The next steps involved trypsin digestion of the proteins, labeling of the resulting peptides with the iTRAQ reagents, pooling samples in a single mix and fractionation of the labeled peptides by SCX. The 27 initial fractions were collected and their peptide complexity was determined by MALDI-TOF MS. Low complexity fractions were pooled, given a total of 12 final fractions. These 12 fractions were subjected to nanoHPLC-MS/MS in an Orbitrap Velos hybrid mass spectrometer. Spectra were analyzed with ProteinPilot leading to the identification of 247 proteins with at least 2 peptides (315 proteins with a single peptide) (Table S-1 in the Supplementary Material). An experiment with 300 μ g of depleted serum treated in the same conditions except for iTRAQ labeling, led to the identification of 235 proteins (results not shown). This result indicates that iTRAQ labeling with the analytical conditions used in this study does not substantially modify peptide ionization efficiency and protein identification. This finding is relevant because the effect of iTRAQ labeling seems to depend on the analytical system used. For example, iTRAQ labeling increases the number of identified proteins when using MALDI-TOF-TOF [33], but decreases the number with pulsed-Q dissociation (PQD) on a dual-pressure linear ion trap LTQ Velos [34] or with CID on a non-dual-pressure linear ion trap [35]. CID shows better performance for identification on linear ion traps, so quantification methods that use isobaric tags usually combine CID fragmentation for identification with PQD or HCD scans for quantification [36-38]. We have achieved similar protein identification for 8-plex labeled samples than for label-free samples, showing that the combination of dual-pressure LIT CID for identification with HCD for quantification, as implemented in the Orbitrap Velos hybrid mass spectrometer, is advantageous for isobaric tag quantification workflows.

Gene ontology analysis of the identified proteins showed that most of them had an extracellular location (Figure 3a), as expected. The main activities were defined by functional classification as binding, catalytic and enzyme regulator (Figure 3b).

Quantitative analysis

The iTRAQ label ratios were used for relative quantification of 289 proteins, 237 of them with at least two peptides (Table S-2 in the Supplementary Material). Comparison of protein abundance between responder and non-responder patients was used to identify potential biomarkers for prediction of response to infliximab. Statistical significance rather than fold difference is preferred for selection of proteins showing differential abundance [39]. Therefore, we have used p-values < 0.05 from t-test comparisons between the two groups of patients to discover 14 differential proteins (Table 1 and Figure S-1 in the Supplementary Material). As expected, the AUC values derived from ROC analyses were concordant with the p-values from the t-tests: with the most discriminant proteins being identified by the two analyses (Table 1 and Table S-2 in the Supplementary Material). Each value of AUC can be interpreted as the probability that the biomarker will rank a randomly chosen non-responder higher than a randomly chosen responder [28]. The AUC value for a perfect test is 1.0, whereas the value for a completely random test is 0.5. It is commonly accepted that tests with AUC below 0.75 are unlikely to have interest for clinical use, whereas those with values over 0.75 could be of utility. All the differential proteins in our study were in this later group with high predictive potential (Table 1) and compare favourably with those previously identified for prediction of response to treatment in RA [8], with other predictors of response [40-42] and with other predictors in RA research [43-45]. All the differential proteins, except for adipocyte plasma membrane-associated protein (APMAP), have been previously related to RA or to the inflammatory response. These relationships with inflammation and the disease process could explain that all of them were more abundant in the non-responder subset of patients than in the responder group. In addition, as the serum samples were obtained before the start of infliximab treatment, all of them came from patients with active inflammation because this is a requirement for the prescription of this drug. The fold difference values should be interpreted considering that iTRAQ quantification underestimates the abundance differences [46],

1 and therefore some of the results may be possibly more disparate between the two
2 groups of patients when assessed with other techniques.

3 Results of pathway analysis showed that the differential proteins were
4 significantly enriched in the inflammatory response and protein synthesis networks
5 (scores 46 and 41, respectively). These results highlight a potential role for
6 apolipoproteins and complement factors in the differential response to infliximab
7 (Figure 4). Among the apolipoproteins, three were significantly more abundant in non-
8 responder patients: apoB-100, apoA-II and apoM. In addition, apoA-I, apoC-I and
9 apoC-II were near the significance threshold (P values < 0.08). Proteins of this family
10 bind lipids to form lipoprotein particles and transport these lipids to tissues. ApoB-100
11 is the hepatic isoform of apoB. It is the main protein component of low-density
12 lipoproteins (LDL) that carry most of the cholesterol that is transported to the tissues.
13 ApoB is the recognition signal for the cellular binding and internalization of LDL, and it
14 is a very good biomarker for cardiovascular risk [47]. ApoA-I and apoA-II are the main
15 apolipoproteins of high-density lipoproteins (HDL). ApoA-I is associated with
16 protection from cardiovascular risk because it promotes cholesterol efflux from tissues
17 to the liver for excretion. It has been much more extensively studied than apoA-II,
18 which seems to play a crucial role in triglyceride catabolism [48]. ApoM is also a HDL-
19 associated apolipoprotein with anti-atherogenic potential probably through an
20 antioxidant effect [49]. ApoC-I is a component of HDL and is also associated with
21 triglycerides and cholesterol in very low density lipoproteins (VLDL). ApoC-II is also a
22 component of VLDL, and it is incorporated to these particles once they are circulating
23 in the blood. The differences we have found in apolipoproteins should be considered in
24 the context of the complex interactions between chronic inflammation and lipid
25 metabolism in RA that we still do not completely understand. An increased mortality of
26 RA patients due to cardiovascular events has been reported [50]. This increase is partly
27 due to the adverse serum lipid profile found in untreated patients, low levels of HDL
28 cholesterol and increased LDL/HDL cholesterol and apoB/apoA-I ratios [51,52]. This
29 adverse lipid profile could be partially explained by changes in the expression of genes
30 in the liver or in the reticuloendothelial system as part of the acute phase response. In
31 addition, inflammation induces changes in the composition of the HDL and LDL
32 particles and in the clearance of cholesterol [52,53]. The lipid profile improves during
33 effective treatment, probably as consequence of the control of inflammation [54,55].
34 Together with these effects of inflammation on apolipoproteins there are also effects of

1 apolipoproteins on inflammation. The most studied effects have been those of apoA-I,
2 which is anti-inflammatory [56], and apoB, which is proinflammatory [57], but it has
3 been also reported that apoA II has an anti-inflammatory role [58] and apoC-I a
4 proinflammatory one [59]. These contrasting effects pose a problem for a simple
5 interpretation of our results because all the differential apolipoproteins showed higher
6 levels in the non-responder group than in the responder one. However, recent evidence
7 indicates that the known roles of apolipoproteins are reversed in a subset of RA patients
8 that have proinflammatory HDL particles in place of anti-inflammatory HDL, due to
9 changes in composition [60]. This change of properties has been shown to be a
10 consequence of the acute phase response [61] and might mean that all the observed
11 differences in our study are directed towards a more damaging and proinflammatory
12 lipid profile.

13 Another protein related with lipid metabolism in our significant findings is
14 APMAP. This protein is a transmembrane protein necessary for adipocyte
15 differentiation that is increased in obesity models [62], although no relationship with
16 inflammation or RA have been described.

17 Regarding the other overrepresented functional pathway, there were five
18 complement-related proteins among the proteins showing significant differences: C4B-
19 alpha chain, complement factor H-related protein 4 (CFHR4), mannan-binding lectin
20 serine protease 2 (MASP2), and inter alpha trypsin inhibitor heavy chain H1 (ITIH1)
21 and H2 (ITIH2). Other four proteins in this pathway showed fold differences over 1.5
22 but were not significantly different: C8 beta, C8 alpha, C5 and complement factor H-
23 related protein 3 (CFHR3). C4B is one of the two isotypes of C4, each of them encoded
24 by a different gene. Upon secretion, the C4 molecules are cleaved in three chains that
25 remain together as a trimer. The classic and the lectin complement activation pathways
26 include cleavage of the C4 alpha chain, which we have found different, into the C4a
27 anaphylotoxin and C4b, which continues with the activation cascade. The different
28 complement activation pathways converge in C3, whose activation is followed by the
29 cleavage of C5 in C5a, another anaphylotoxin, and C5b, which initiates the membrane
30 attack complex. This complex is the effector cytolytic endproduct of the complement
31 system and includes, among others, complement factors C5b and C8. The latter is made
32 of three subunits encoded in separated genes: C8 alpha, beta and gamma. CFHR4 is a
33 member of the CFH family that has recently been identified as promoting complement
34 activation via the alternative and classic pathways [63]. CFHR3 is a member of the

1 same family that seems to be involved in regulation of the complement system by
2 inhibiting the activation of C3 [64]. MASP2, in turn, is a serum protease that activates
3 the lectin pathway via the cleavage of C4 and C2 [65]. Finally, the inter alpha trypsin
4 inhibitor (I α I) complex is made of two heavy chains ITIH1 and ITIH2 plus bikunin, and
5 it is a broad spectrum proteinase inhibitor. Among its many targets, it inhibits the early
6 phases of complement activation by the three pathways: classic, alternative and lectin
7 [66]. All these differential proteins related to complement can have a role in RA, since
8 the complement system is activated in the inflamed joints of patients with RA. This
9 activation state has been shown by the relative consumption of C3 and C4 in the
10 synovial fluid together with increased concentrations of C5a and the membrane attack
11 complex [67]. The higher abundance of all these proteins in the non-responder patients
12 makes it tempting to propose that this subset of patients is characterized by more
13 complement activation. However, this interpretation should be very cautious because
14 regulation of the complement system is mainly done at the activation levels and not at
15 the transcription level, and because ITIH1 and ITIH2 are inhibitors of complement
16 activation. An additional element to consider is that many components of the
17 complement system are acute phase reactants and they could be increased as a
18 consequence of inflammation [68].

19 Other differential proteins in our results were also part of the acute phase
20 response: ceruloplasmin (CP), thrombospondin (THBS1), vitamin D-binding protein
21 (GC) and fibronectin (FN1). CP is a ferroxidase enzyme involved in iron transport and
22 metabolism that has been proposed as a link between iron metabolism and the immune
23 system [69]. THBS1 is a multifunctional protein that mediates cell-to-cell and cell-to-
24 matrix interactions. It has been related to RA pathogenesis via the activation of
25 transforming growth factor beta (TGF β), and more generally to acute and chronic
26 inflammation through a variety of mechanisms [70]. GC is involved in the transport of
27 vitamin D and its metabolites to target tissues [71]. It is also the precursor of vitamin D-
28 binding protein-derived macrophage-activating factor (GcMAF) that results from
29 enzymatic modifications taking place in B and T cells [72]. GcMAF is a potent activator
30 of monocyte and macrophage functions that has not yet been specifically studied in RA.
31 Finally, FN1 is one of the most abundant proteins in inflamed joints of patients with RA
32 [73], and it is also related with RA as citrullinated FN1. This posttranscriptional
33 modification and antibodies against it are present in synovial tissue, synovial fluid and
34 sera of RA patients [74]. In this regard, it is interesting to note that protein citrullination

1 and anti-citrullinated protein antibodies play a central role in the pathophysiology of RA
2 [75]. Most of the proteins in this group of acute phase reactants have been found at
3 elevated levels in serum of patients with RA [15,73,76]. However, it is uncertain if their
4 higher level in the non-responder patients of our study could be ascribed to a more
5 severe inflammation. The reasons for this doubt are that clinical studies have not
6 identified any sign of inflammation as predictive of response to infliximab [77], and that
7 other prominent acute phase reactants, serum amyloid A protein and C-reactive protein,
8 showed a non-significant excess in the responder group (fold differences 0.8 and 0.65,
9 respectively). Therefore, we cannot exclude the possibility that the higher abundance of
10 these proteins is due to specific mechanisms unrelated with the intensity of
11 inflammation.

12 Finally, gelsolin (GSN) is a protein that does not fit in any of the previous
13 groups. In blood, it contributes to limit inflammatory responses by acting as a scavenger
14 binding the actin released by tissue damage, bioactive lipids and proinflammatory
15 mediators [78]. It is decreased in multiple acute and chronic inflammatory diseases
16 including RA [79]. Possible mechanisms of this decrease include clearance of the GSN
17 complexes and trapping of GSN-actin into the inflamed joints in RA. As commented for
18 the other groups of proteins, it is impossible to know at this stage what could be behind
19 the higher levels of GSN found in the non-responder group of patients.

20 The only previous study with similar aims has been already mentioned [8]. It
21 identified six potential proteins that were different between the non-responder and
22 responder RA patients treated with infliximab. These proteins showed AUC ranging
23 from 0.761 to 0.846, but not statistical tests or fold differences were reported. Five of
24 them were more abundant in the non-responder group and only one in the responder
25 patients, which is a direction of differences similar to our findings. Four of the proteins
26 were not further characterized because the authors used SELDI-TOF-MS and needed
27 purification for identification. The two identified proteins were also present in our
28 analysis. Platelet factor 4 was more abundant in the non-responder patients in both
29 studies (fold difference NR/R = 1.39; AUC = 0.81 in our samples), but the difference
30 was not significant in our analysis ($P = 0.2$). It has been also highlighted as a biomarker
31 for prediction of response to infliximab in a Crohn's disease study showing also higher
32 concentration in the non-reponder patients [80]. Therefore, platelet factor 4 seems a
33 good candidate biomarker although it did not came in the top list of our study. The
34 second protein, apoA-1 showed contrasting results. It was almost significantly more

abundant in the non-responder patients in our study, as already discussed, whereas it was more abundant in responder patients than in non-responder ones in Trocmé et al. [8]. This discordant result serves us to remind that all these results need to be replicated in new studies because of the exploratory nature of the previous and the current studies.

Conclusions

This report is the first label-based quantitative proteomics study aimed at discovering potential serum biomarkers for prediction of response to biologics in RA. The results have been encouraging in several respects: the number of identified proteins, the dynamic range of concentrations they covered, and the number of differential proteins between the two groups compared. The panel of differential proteins needs further verification and clinical validation, but already it has been possible to notice that most of these proteins are known to be related with important processes in RA and many of them are known to show altered levels or function related with RA or chronic inflammation. Therefore, we expect that they will help establish approaches to classify RA patients as responders and non-responders to infliximab before the beginning of the treatment, in order to treat anticipated non-responders with an alternative drug.

Conflict of interest statement

All authors declare there are no financial/commercial conflicts of interest.

Acknowledgments

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Figure captions.

Figure 1. Discovery-driven experimental workflow of the study. R, responder; NR, non-responder; CID, collision-induced dissociation; HCD, higher-energy collisional dissociation.

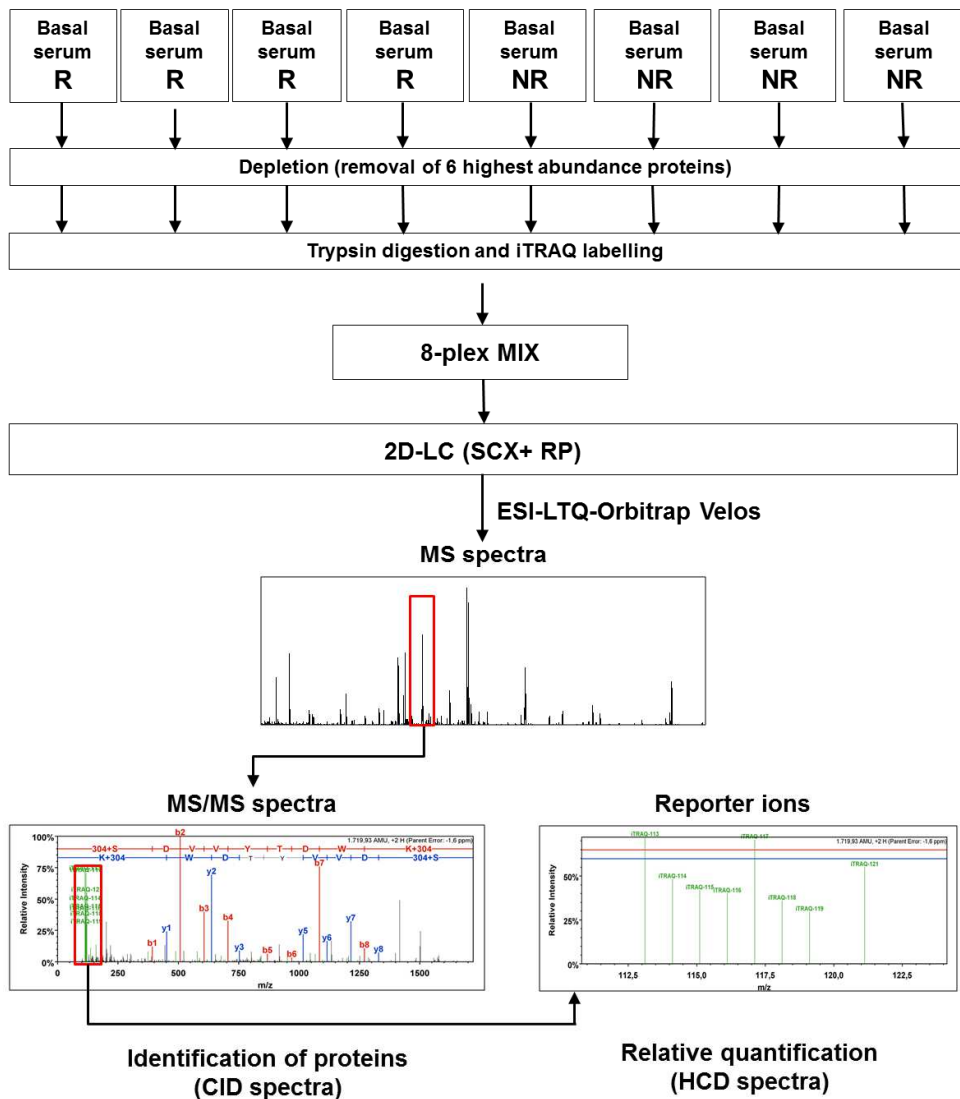
Figure 2. Depletion of the 6 highest abundance proteins from serum. 10% NuPAGE Bis-Tris 1-DE gel image of the different protein fractions.

Figure 3. Gene ontology (GO) annotation of identified serum proteins according to (a) cellular location; and (b) molecular function.

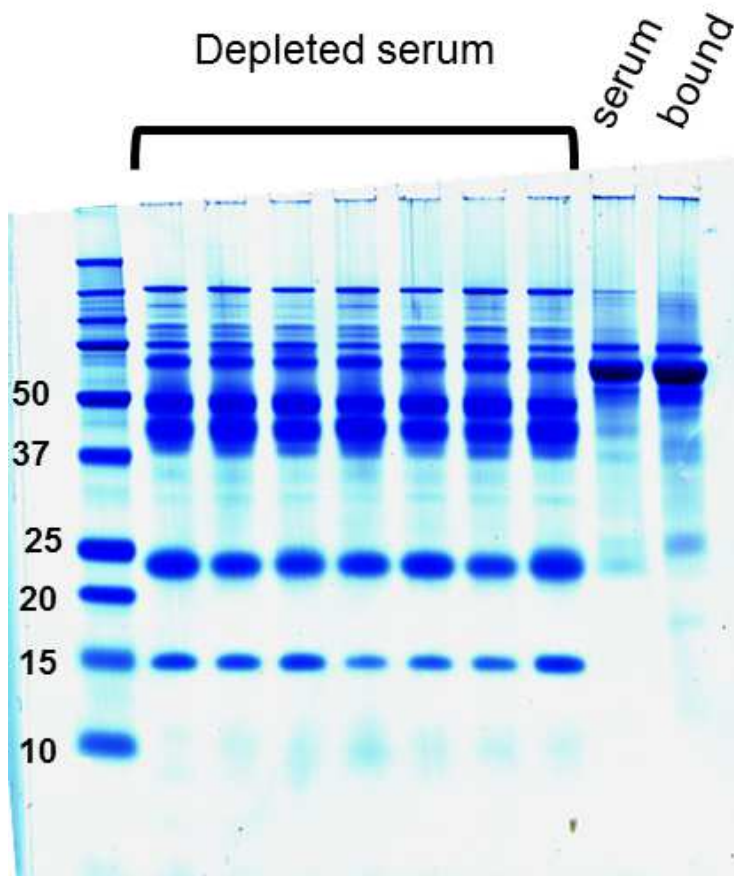
Figure 4. Pathway analysis of proteins that showed differential abundance between responder and non-responder patients with RA treated with infliximab. Direct interactions are shown. Proteins with R/NR ratios > 1.5 are in green, and with NR/R ratios > 1.5 are in red. Merged two top score networks are shown (immune and inflammatory response, score 46, and protein synthesis, score 41), centered on low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as reflected by the number of interactions.

Table 1. Serum proteins showing differential abundance between the responder and non-responder patients with RA treated with infliximab.

Accession number	Protein name	Non-responder/Responder	p-val	AUC
D6RAK8	Vitamin D-binding protein splicing variant GC-006	5.359	0.0386	1
P00450	Ceruloplasmin	3.375	0.0178	1
P04114	Apolipoprotein B-100	2.198	0.0348	0.938
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	2.072	0.0230	1
P07996	Thrombospondin-1	1.994	0.0319	1
Q6U2E9	Complement C4-B alpha chain	1.971	0.0315	0.938
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	1.785	0.0388	0.938
P06396	Gelsolin	1.718	0.0328	1
P02652	Apolipoprotein A-II	1.718	0.0414	1
P02751-7	Fibronectin isoform 7	1.682	0.0340	0.938
C9J7J7	Complement factor H-related protein 4	1.680	0.0486	0.875
O95445	Apolipoprotein M	1.601	0.0325	1
Q9HDC9	Adipocyte plasma membrane-associated protein	1.508	0.0219	1
O00187	Mannan-binding lectin serine protease 2	1.336	0.0246	0.938

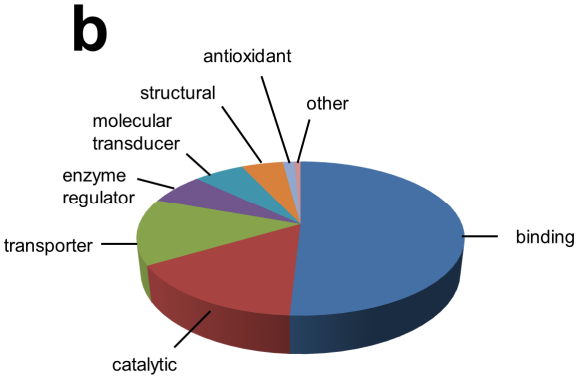
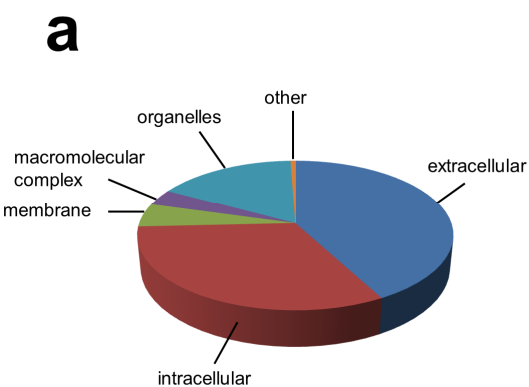


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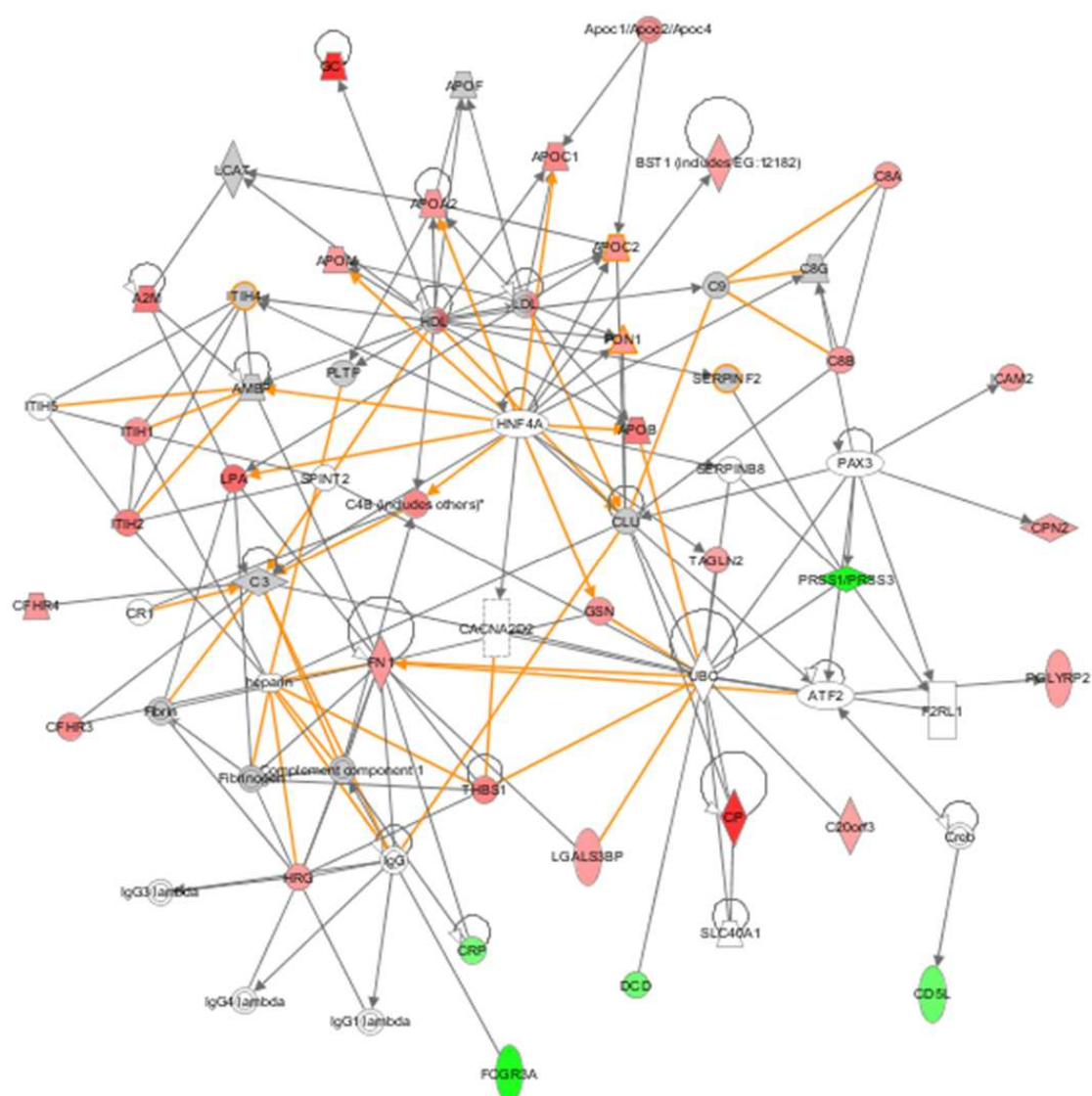
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